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The *Drosophila* Female Sterile Mutation *twin peaks* Is a Novel Allele of *tramtrack* and Reveals a Requirement for Ttk69 in Epithelial Morphogenesis

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The Drosophila gene *tramtrack* (*ttk*) encodes two transcriptional repressors, Ttk69 and Ttk88, which are required for normal embryogenesis and imaginal disc development. Here, we characterize a novel female sterile allele of *tramtrack* called *twin peaks* (*ttk*^{twk}) that, unlike other *tramtrack* alleles, has no effect on viability and produces no obvious morphological defects, except during oogenesis. Females homozygous for *twin peaks* produce small eggs with thin eggshells and short dorsal respiratory appendages. Complementation analyses, immunolocalization, and rescue data demonstrate that these defects are due to loss of Ttk69, which is expressed in the follicle cells and is required for normal chorion production and dorsal follicle-cell migration. Analyses of phenotypes produced by mutations in other loci that regulate eggshell synthesis suggest that the chorion production and follicle-cell migration defects are independent. We present evidence that *twin peaks* disrupts a promoter or promoters required for late-stage follicle-cell expression of Ttk69. We hypothesize that loss of Ttk69 in all follicle cells disrupts chorion gene expression and lack of function in dorsal anterior follicle cells inhibits morphogenetic changes required for elongating the dorsal appendages.

***Key Words: tramtrack;* oogenesis; dorsal appendages; epithelial morphogenesis.**

INTRODUCTION

A central goal of developmental biology is to understand how complex three-dimensional structures emerge from much simpler forms. One universal mechanism for the generation of complexity is the morphogenesis of flat epithelial tissues into alternative shapes. In metazoans, gastrulation, organogenesis, and wound healing are all rooted in the movement of sheets of cells. Examples of this process include kidney tubule development, breast duct formation, and lung morphogenesis, which depend on the reorganization of epithelial sheets into tubes of cells (Cunha, 1994; Hogan *et al.*, 1997; Lechner and Dressler, 1997). Many elegant studies have identified molecules that are required for the establishment, organization, and maintenance of epithelia (Drubin and Nelson, 1996; Eaton and Simons, 1995; Rodriguez-Boulan and Nelson, 1989; Tepass, 1997). In general, however, much less is known about the genetic pathways regulating these molecules and the link between cell fate-determining processes and cytoskeletal functions.

We are using dorsal appendage morphogenesis in *Drosophila* oogenesis as a model system for studying the relationship between patterning and morphogenesis. Each of the two dorsal respiratory appendages of the *Drosophila* egg chamber is formed by secretion of eggshell proteins into a tube of follicle cells. This tube is generated by cell shape changes and rearrangements within an epithelial sheet (Dorman and Berg, unpublished observations). Dorsal appendage formation is therefore similar to more complicated examples of organogenesis. In addition, the study of dorsal appendage formation provides several advantages that make it an excellent system for investigating the regulation of epithelial morphogenesis. First, the signaling events that determine two populations of dorsal follicle cells are well understood (Peri *et al.*, 1999; Ray and Schüpbach, 1996; Wasserman and Freeman, 1998). This understanding facilitates our ability to uncouple effects on patterning from morphogenesis. Further, powerful genetic tools, including mutations that disrupt dorsal appendage formation, have

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allowed us to begin to unravel the genetic circuitry underlying the regulation of epithelial morphogenesis.

The Drosophila egg chamber contains 16 interconnected germline cells, consisting of 1 oocyte nourished by 15 highly polyploid nurse cells; these germline cells are surrounded by a monolayer of ~1000 somatic follicle cells (Spradling, 1993). The nurse cells synthesize components required by the developing oocyte and future embryo, then transport these components into the oocyte through cytoplasmic bridges called ring canals. The follicle cells secrete the chorion that makes up the three layers of the eggshell: the vitelline envelope, the endochorion, and the exochorion. A subset of these follicle cells undergoes morphogenesis to generate the dorsal appendages, specialized structures that facilitate gas exchange in the developing embryo (Hinton, 1969).

At stage 10 of oogenesis, the oocyte occupies the posterior half of the egg chamber, the nurse cells the anterior half, and the oocyte nucleus is positioned at the dorsal anterior corner of the oocyte. The majority of follicle cells forms a columnar layer over the oocyte, while a few follicle cells are stretched out over the nurse cells. During stage 10B, those follicle cells closest to the nurse cell/oocyte boundary begin to migrate centripetally, between nurse cells and oocyte. The centripetal cells secrete the operculum, which is a thin layer of chorion that functions as an escape hatch for the larva, the collar, which is a hinge on which the operculum swings, and the micropyle, a cone-shaped structure through which the sperm enters (Spradling, 1993).

Shortly after centripetal migration (stage 10B), the nurse cells rapidly transfer their contents into the oocyte (stage 11) then begin to degenerate and undergo apoptosis (stages 12–14). At the same time, two groups of approximately 65–80 anterior, dorsal follicle cells, one on each side of the dorsal midline of the egg chamber, migrate over the nurse cells, laying down the chorion of the two dorsal appendages.

Extensive studies have defined the signaling events that determine two populations of dorsal follicle cells. Dorsal follicle-cell fate determination begins when transcripts encoding the TGFα-like signaling molecule Gurken (Grk) become localized in a cap above the oocyte nucleus. Grk signals via the epidermal growth factor receptor homologue (Egfr) to the follicle cells, activating a signal transduction cascade involving the Ras/Raf/MAPK pathway (Ray and Schüpbach, 1996). This initial signaling event defines a set of dorsal anterior follicle cells and induces a second signaling cascade involving three additional Egfr ligands. This second cascade amplifies and refines the initial Grk signal, leading to the definition of two separate populations of dorsal follicle cells (Peri *et al.*, 1999; Wasserman and Freeman, 1998). These events are required for the production of two separate dorsal appendages. Disruptions of this process result in dorsalization or ventralization of the follicular epithelium and the eggshell. Partial ventralization generally results in failure to determine two separate populations of cells, leading to the production of a single dorsal appendage at the dorsal midline. Complete ventralization results in the absence of dorsal cell fates and the concomitant loss of dorsal appendages (Van Buskirk and Schüpbach, 1999).

Information along the anterior–posterior axis also contributes to cell-fate determination within the dorsal appendage primordia. The BMP2/4 homologue encoded by *dpp* is expressed in the stretch cells and a single row of centripetally migrating cells. This morphogen radiates posteriorly and alters columnar cell fates (Twombly *et al.*, 1996). High levels of Dpp repress dorsal identities and specify operculum; moderate levels synergize with Grk to define dorsal, while low levels of Dpp are insufficient to allow cells to respond to Egfr signaling (Deng and Bownes, 1997; Dequier *et al.*, 2001; Peri and Roth, 2000; Queenan *et al.*, 1997). Thus, loss-of-function mutations generate short, often paddleless appendages, while overexpression either expands the operculum at the expense of appendage material or creates multiple, often antler-shaped dorsal structures.

The subsequent events underlying dorsal appendage morphogenesis are only beginning to be understood. Analyses of cultured wild-type egg chambers have revealed several phases of dorsal appendage morphogenesis (Dorman *et al.*, unpublished observations). From stages 10B to 12, two groups of dorsal anterior follicle cells move out from the follicular epithelium to form short tubes. Each tube extends forward over the nurse cells, secreting chorion proteins that make up the cylindrical stalk of the dorsal appendage (Fig. 1C). Cells at the anterior end of the tube change shape to produce the flattened paddle of the distal dorsal appendage (Fig. 1E). Finally, upon oviposition, the entire follicular epithelium sloughs off, leaving behind the chorionic structures.

Here, we have used the excellent genetic and cell biological tools available in *Drosophila* to investigate the function of the gene *tramtrack (ttk)* in the regulation of these processes. We describe the isolation and characterization of a novel allele of *ttk*, ttk^{twk}. Unlike other *ttk* alleles, which usually result in lethality, *twk* is completely viable but female sterile, producing small, round eggs with a severe defect in chorion production. In addition, the dorsal follicle cells initiate tube formation but fail to move anteriorly, resulting in the synthesis of rudimentary nubs in place of dorsal appendages. We show that the *ttk* isoform Ttk69 is expressed in the follicle cells and is required for dorsal appendage morphogenesis. In addition, we show that Ttk69 regulates morphogenesis and is not required for cell fate determination.

MATERIALS AND METHODS

Fly Strains and Genetics

Fly culture and crosses were performed according to standard procedures. All crosses were performed at 25°C, except rescue crosses, which were performed at 18°C. The ttk^{twk} mutation results

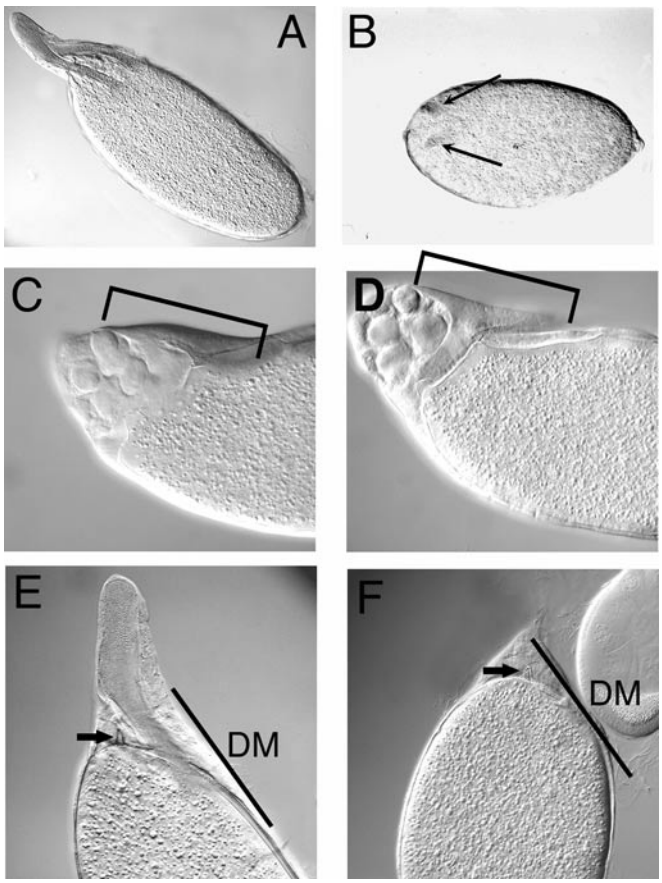

FIG. 1. *twk* egg chambers initiate, but fail to complete, dorsal appendage morphogenesis. In (A), anterior is upper left. In (B–D), anterior is to the left; dorsal is up. In (E) and (F) anterior is up. (A, C, E) Canton S. (B, D, F) *twk*. (A) A mature (stage 14) wild-type egg chamber. Anterior is upper left. The dorsal side of the egg chamber is marked by the dorsal appendages, which extend anteriorly from the main body of the egg. (B) A stage-14 *twk* egg chamber. The arrows indicate the mutant dorsal appendages. While two appendages initially formed with proper spacing, these appendages failed to extend to their normal length (compare with A). Note also the abnormally short and round shape of the egg. (C) A wild-type stage-12 egg chamber. At this stage, the developing dorsal appendage is apparent as a wedge of cells (bracket) present between the oocyte and nurse cells. Chorion proteins can be seen as a line between the two faces of the wedge. (D) A stage-12 *twk* egg chamber. Although chorion proteins are not being deposited normally (compare D with C), *twk* follicle cells initiate morphogenesis. The degree of cell movement (marked by the bracket) is similar to wild type at this stage. (E) A wild-type stage 13 egg chamber. By this stage, appendage morphogenesis is nearly complete. DM, dorsal midline; small arrow, micropyle. (F) A stage 13 *twk* egg chamber. *twk* egg chambers display a severe defect in follicle cell movement, and the dorsal appendages have not extended past the degenerating nurse cells at the anterior end of the egg chamber. DM, dorsal midline; small arrow, micropyle.

from a *P[lacZ; ry$^+$]* insertion and was isolated as a recessive female sterile mutation [*fs(3)07223*] in a large-scale *P*-element mutagenesis screen (Karpen and Spradling, 1992).

We used the following fly strains: Canton S, *ry^{506} ttk^{twk}/TM3, ry^{RK} Sb e, y w; mwh ry^{506} e ttk^{twk}/TM3, ry^{RK} Sb e, y cv dec2^1 v f/FM0, y^{3Id} sc^8 w v m^2 f B* (obtained from the Bloomington Stock Center), *y; Df(3R)awd-KRB (100D1; 100D3-4)/TM3, y$^+$ Sb Ser, w; ry^{506} ttk^{lell}/TM3, ry^{RK} Sb e*, (Xiong and Montell, 1993), *ry^{506} ttk^1/TM3, y$^+$ Sb Ser* (Xiong and Montell, 1993), *w; hs-GAL4* (Brand *et al.*, 1994), *w UAS-ttk88* (Giesen *et al.*, 1997), *w; UAS-ttk69* (Giesen *et al.*, 1997), *w; hs-GAL4 e ttk^{twk}/TM3, w UAS-ttk88; ry^{506} ttk^{lell}/TM3, ry^{RK} Sb e*, and *w; UAS-ttk69 ttk^{lell}/TM3, ry^{RK} Sb e*. We examined the β-galactosidase expression patterns of three *PZ* enhancer trap lines in *twk* ovaries to test the specificity of the effect on chorion-gene expression and dorsal follicle-cell fate: (1) *l(3)02255 ry^{506}/TM3, ry^{RK} Sb Ser* (all FC, weak, and S12–14 dorsal appendage FC, strong), (2) *numb^{03235}/CyO; ry^{506}* (S13 dorsal appendage FC), and (3) *l(2)08492/CyO; ry^{506}* (S14 operculum and dorsal appendage FC).

We employed Δ2-3 transposase (Robertson *et al.*, 1988) to generate *P*-element excision alleles as described previously (Rittenhouse and Berg, 1995). Briefly, we crossed males homozygous for the *mwh ry^{506} e ttk^{twk}* chromosome to virgin females of the genotype *Sp/CyO; ry Sb Δ2–3/TM6, Ubx*. From this cross, we collected 100 males of genotype *mwh ry^{506} e ttk^{twk}/ry Sb Δ2–3* and crossed them, 2 per vial, to *ry^{506}/TM3, ry^{RK} Sb e* virgin females. From each of these 50 vials, we identified 2 ry$^-$ sons and created stocks by balancing the ry$^-$ chromosome through crosses with *TM3, ry^{RK} Sb e* virgin females.

For the rescue experiments, we crossed females of genotype *w; hs-GAL4 e ttk^{twk}/TM3, ry^{RK} Sb e* to *w UAS-ttk88; ry^{506} ttk^{lell}/TM3, ry^{RK} Sb e* or *w: UAS-ttk69 ttk^{lell}/TM3, ry^{RK} Sb e* males. The female progeny of genotype *w UAS-ttk88/+; hs-GAL4 e ttk^{twk}/ttk^{lell}* or *w; hs-GAL4 e ttk^{twk}/UAS-ttk69 ttk^{lell}* were aged for 1–2 days, then subjected to one of a variety of heat-shock regimens. Files were heat shocked for 10, 20, 30, or 45 min at 30, 35, or 37°C. Heat shock was performed in vials with no food. After heat shock, flies were placed without anesthetization in fresh, yeasted vials with males and allowed to recover for 20 h before their ovaries were dissected. Flies were paralyzed and died if anesthetized after heat shock-induced expression of Ttk69. Dorsal appendages were scored as "partially rescued" if they extended past the nurse-cell material at the anterior end of the egg chamber. We defined the frequency of rescue as the total number of partially and completely rescued eggs divided by the total number of stage 13 and 14 egg chambers.

Egg Chamber Examination Procedures and DAPI Staining

Ovaries were dissected in 1× PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4) and fixed in 4% EM-grade formaldehyde (Ted Pella, Inc) in PBS for 40 min. After washing in 1× PBT (PBS + 0.2% Tween 20), the ovaries were incubated for 1 h in a 0.2-μg/ml solution of 4′-6′-diamidino-2-phenylindole (DAPI) in PBT. Ovaries were then washed twice in PBT and mounted in 70% glycerol/30% PBS.

All microscopy was performed on a Nikon Microphot FXA equipped with differential interference contrast and epifluorescent optics.

PCR Analysis of Excision Lines

For each excision line analyzed, genomic DNA was prepared from homozygous flies. PCR was performed on DNA from the equivalent of 1/3 fly. We used the following primers: IR (specific to the *P*-element inverted repeat): 5′-GCTCTAGACGGGACCAC-

TABLE 1

Percent of Egg Chambers at Different Stages of Oogenesis Displaying Normal Morphology

Maternal genotype	Stage	Normal egg chambers/total	Normal morphology (%)
Canton S	10b	52/52	100
	11	5/5	100
	12	28/29	96.5
	13	20/20	100
	14	28/28	100
twk/twk	10b	44/44	100
	11	10/10	100
	12	4/47	8.5
	13	1/30	3.3
	14	0/59	0

CTTATGT-3′; Primer A: 5′-AGCCCCATTCAAATGTTCG-3′; Primer A′: 5′-CTGAGCTGGAATAGCATGG-3′; Primer B′: 5′-GACGCAGAAGCCATTTTACC-3′. PCR conditions specific for each reaction are available on request.

Southern Analysis

Genomic DNA was isolated from male and female flies by standard methods. DNA from five flies was digested with *Eco*RI or *Sal*I (New England Biolabs) according to the manufacturer's recommendations, size fractionated on 1% agarose gels, and transferred to Hybond-N nylon membrane (Amersham). Blots were hybridized with digoxygenin-labeled DNA probes (generated by random priming) in a hybridization solution containing 50% formamide. Plasmids used to generate probes were: p104.40, containing a 4.0-kb *Eco*RI fragment from the X-chromosome chorion gene cluster (Spradling, 1981), and pYES-3.8S, containing a 3.8-kb *Sal*I fragment from the third-chromosome chorion gene cluster (Lu and Tower, 1997). Hybrids were detected with alkaline phosphatase-conjugated anti-digoxygenin antibody (Roche) using the CDP-Star chemiluminescent reagent (Roche), according to the supplier's protocol. For quantitation, autoradiograms were scanned with an Apple ColorOne Scanner (Apple Computer, Inc.) and imported into Adobe Photoshop (Adobe Systems, Inc.). We calculated the relative intensity of bands compared with background levels using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). We demonstrated that each lane was loaded with an equivalent amount of material by hybridizing with a probe specific for *oskar*, a gene in an unamplified region of the genome. To assure detection within the linear range of the film, we probed a dilution series, then exposed the blot for various times. Two independent experiments gave identical results.

Western Analysis and Immunostaining of Ovaries

For Western blotting, ovaries from 5–10 females were dissected into cold 1× PBS and homogenized. The homogenate was then boiled and an equal volume of 2× sodium dodecyl sulfate sample buffer (100 mM Tris, pH 6.8, 200 mM dithiothreitol, 4% sodium

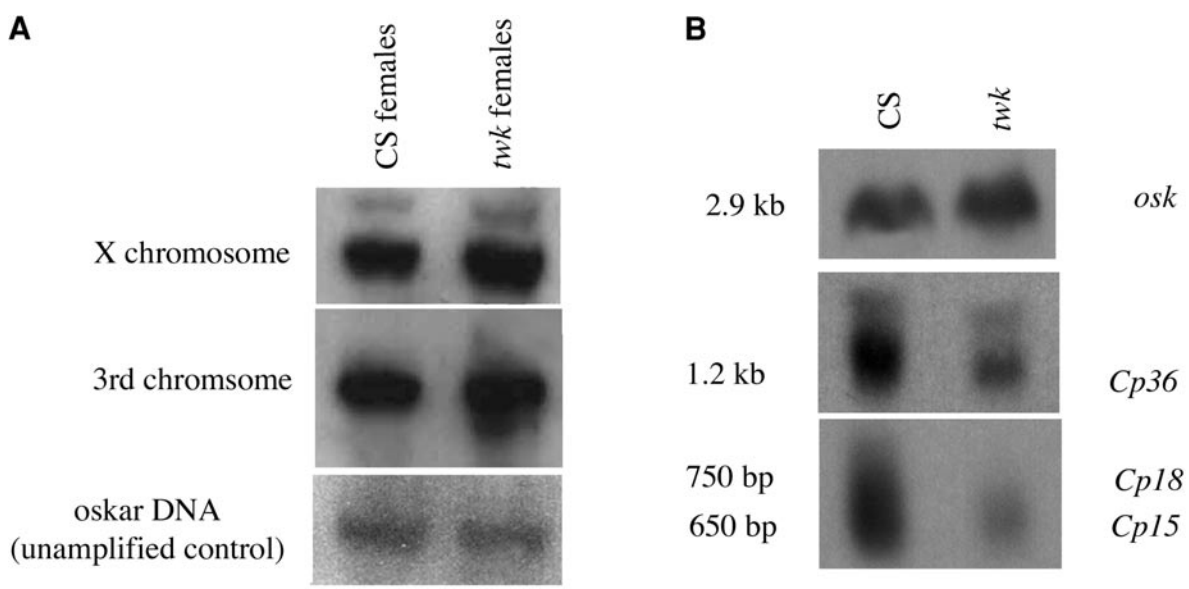

FIG. 2. (A) Southern blot comparing amplification of the X- and third-chromosome chorion gene clusters in Canton S (CS) and *twk* females. Hybridization with a probe for *oskar*, an unamplified gene, is used as a loading control. Levels of both X- and third-chromosome chorion DNA are the same in *twk* and wild-type. (B) Northern analysis of chorion gene transcript levels: Total ovarian RNA from 2-day-old Canton S and *twk* females was hybridized with a DNA probe specific to the X-chromosome chorion-gene *Cp36* and a probe recognizing the third-chromosome genes *Cp18* and *Cp15*. *oskar* mRNA serves as a loading control. We detected considerable reductions in transcript levels for *Cp36* and at least one of the third-chromosome chorion genes.

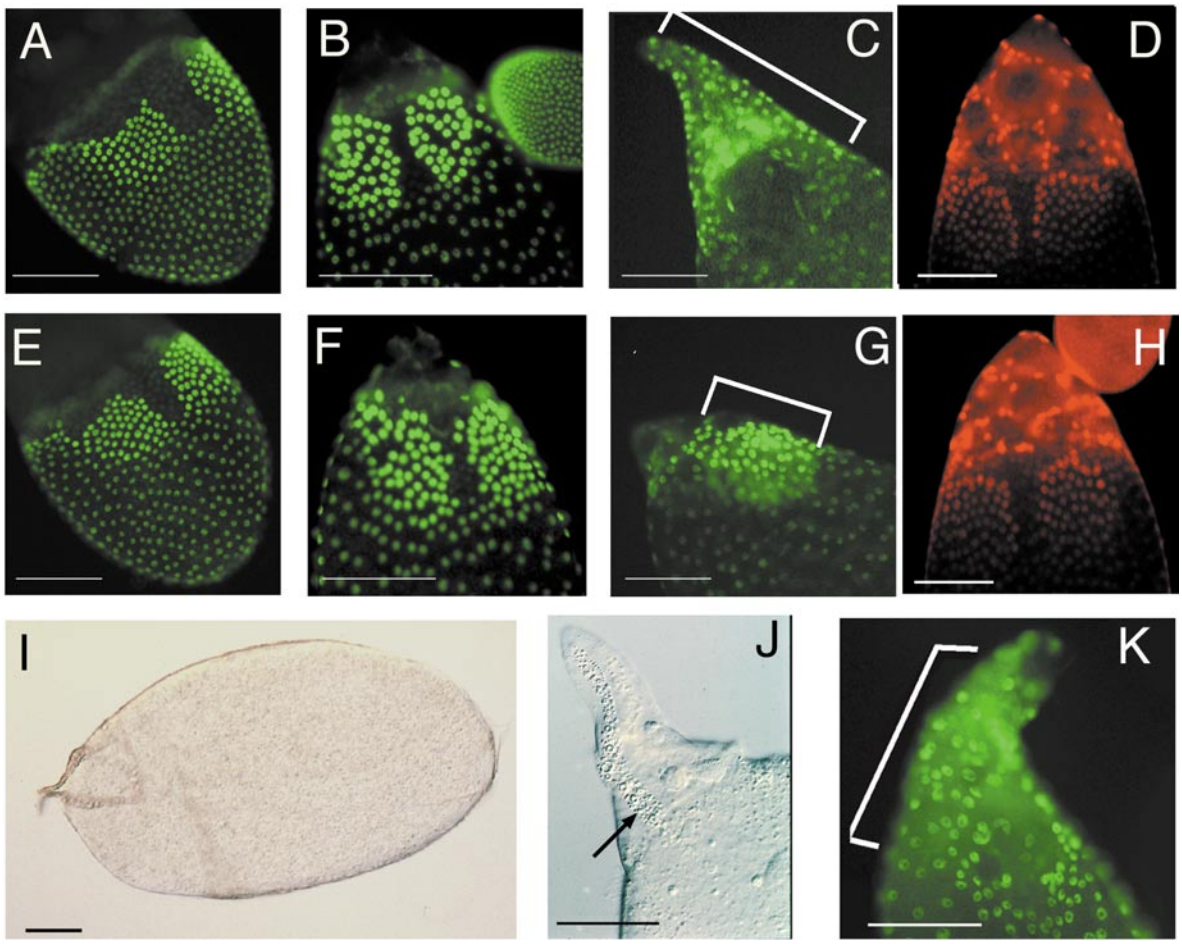

FIG. 3. (A–H) Broad-Complex and Jun expression reveal normal cell fate determination in *twk* dorsal appendage primordia. Anterior is to the top left, except in (D) and (H), where anterior is up, and (G), a side view. (A–D) Canton S. (E–H) *twk*. (A, E) At stage 10B, Br expression is highest in two patches of dorsal anterior follicle cells, moderate in posterior follicle cells, and at much lower levels on the dorsal midline and in an anterior ring of follicle cells. This modulation reflects a response to the Egfr and Dpp signaling pathways. At this stage, Br expression in *twk* is indistinguishable from wild type. (B, F) At stage 12, Br continues to be expressed at high levels in the migratory cells as they begin to move anteriorly. Br expression in *twk* egg chambers is still similar to wild type. (C, G) At stage 13, high-Br expression continues in the dorsal anterior follicle cells and is down-regulated in the rest of the epithelium. Although *twk* mutant cells (G) do not migrate, the number of Broad-positive cells is similar to wild type. Brackets in (C), (G), and (K) delimit the extent of dorsal follicle cell movement. (D, H) At stage 12, Jun is expressed at high levels in the follicle cells over the nurse cells and in two groups of dorsal anterior follicle cells. Jun expression in *twk* egg chambers is indistinguishable from wild type. (I–K) Chorion production is not required for dorsal follicle cell migration. (I) A stage 14 egg chamber produced by a *dec2/dec2* female. The dorsal appendages are much shorter than those of wild-type eggs (compare with Fig. 1A). Additionally, the egg is shorter and rounder than normal, similar to *twk* egg chambers (compare with Fig. 1B). (J) A magnified view of a dorsal appendage from a *dec2* egg chamber. The chorion material appears perforated (arrow); nevertheless, migration has proceeded to a degree comparable to wild type. (K) A stage 14 *dec2* egg chamber immunostained for Br demonstrates that *dec2* dorsal follicle cells migrate approximately as far as wild type (bracket).

dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol) was added. An amount of protein equivalent to 0.4 ovary was loaded per lane of a 10% polyacrylamide gel. Following electrophoresis, proteins were electroblotted onto Hybond-ECL nitrocellulose membrane (Amersham). Blots were incubated overnight with 1:2000 rabbit anti-Ttk69 antibody (kindly provided by Paul Badenhorst) and 1:10,000 mouse anti-actin antibody (a loading control) (Chemicon), washed, incubated overnight with 1:10,000 each HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (Biorad), and detected using the Renaissance chemiluminescent detection kit (NEN Life Sciences).

For immunostaining, ovaries from 1- or 2-day-old females were dissected into 1× PBS and fixed in 4% EM-grade formaldehyde for 20 min. After rinsing with PBT, ovaries were permeablized for 1 h in PBS plus 1% Triton X-100. Ovaries were blocked in PBT/5% BSA/0.02% sodium azide for 1 h, then incubated overnight in

antibody diluted in the blocking solution. Antibodies were used at the following dilutions: rabbit anti-Ttk69, 1:50; mouse anti-Broad (raised against the Broad-Complex-core domain and kindly provided by Wu-Min Deng), 1:100; and rabbit anti-DJun (kindly provided by Steven Hou), 1:100. Texas Red anti-rabbit (ICN Pharmaceuticals) and Alexafluor 488 anti-mouse (Molecular Probes) secondary antibodies were used at a 1:500 dilution. Egg chambers were mounted in 70% glycerol/30% PBS plus Vectashield anti-fade reagent (Vector Laboratories).

RT-PCR and Northern Analysis

Total RNA was extracted from dissected ovaries or whole male flies by using the guanidinium–acid phenol method (modified from Chomczynski and Sacchi, 1987). For RT-PCR, 10 μg total RNA was primed with 2 pmol primer and reverse transcribed with Superscript II (Gibco BRL), following the supplier's protocol. We used the following primers: for *ttk*, Primer E (used for all *ttk* RT reactions and some PCRs): 5′-GATCGAAGACGGACAGAAGG-3′, Primer A: 5′-AGCCCCATTCAAATGTTCG-3′, Primer B: 5′-CTTTTGCCTTTTCGCAACC-3′, Primer C: 5′-AGTGTTGCAAGCCTTCTTCC-3′, Primer D: 5′-CTGTGGTTGCGTCACTAAGC-3′; for *Broad, Br-C* (used for RT and reverse PCR): 5′-ACAAGATGTTCCATGCAGCC-3′, *Br-Z2:* 5′-TCATCTCCATTTCGCCGGGA-3′ (Tzolovsky et al., 1999). PCR conditions specific for each reaction are available on request.

For Northern blots, 20 μg of total RNA was size fractionated on 1% agarose–formaldehyde gels and transferred to Hybond-N nylon membrane (Amersham). Blots were hybridized with digoxygenin-labeled DNA probes (generated by random priming) in a hybridization solution containing 50% formamide. Hybrids were detected with alkaline phosphatase-conjugated anti-digoxygenin antibody (Roche) using the CDP-Star chemiluminescent reagent (Roche), according to the supplier's protocol. We quantified our results as described above for Southern analysis, normalizing to a signal produced by an *oskar* antisense probe. Three independent experiments produced similar results.

Whole-Mount in Situ Hybridization

In situ hybridization to ovaries was carried out as described (Ephrussi *et al.,* 1991), with the following modifications: hybridization was carried out at 55°C with digoxygenin-labeled antisense RNA probes. Probes were generated by using the DIG-RNA labeling kit (Roche) according to the manufacturer's protocol in a reaction volume of 20 μl. All probes displayed equivalent incorporation of label as assayed by serial dilution dot blots, and all probes were diluted 10-fold into hybridization buffer.

RESULTS

The twin peaks Mutation Affects Dorsal Appendage Morphogenesis

The *twin peaks* mutation [*twk;* also called *fs(3)07223*] was generated in a large-scale *P*-element mutagenesis screen (described in Karpen and Spradling, 1992). Flies homozygous for this mutation are fully viable and display no obvious morphological defects (data not shown). Females homozygous for *twk* are sterile, however, and lay few eggs. Those eggs that are laid display several characteristic

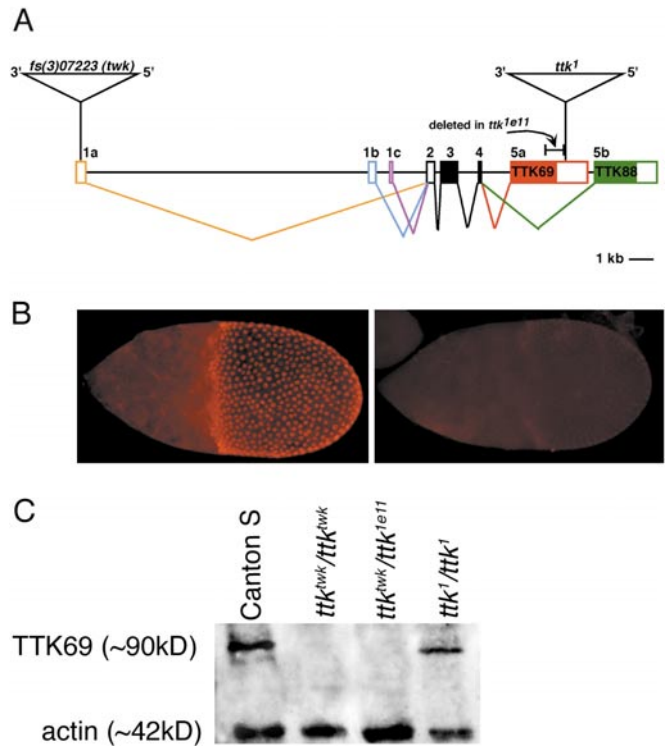

FIG. 4. (A) *tramtrack* genomic structure. Variable exons are shown in color. Open boxes represent UTR; filled boxes represent coding sequence. cDNA analysis reveals three alternative first exons, any of which can be spliced into exon 2. The *twk* mutation is a *P*-element insertion into exon 1a. The ORFs encoding both Ttk69 and Ttk88 begin in exon 3. The 15-kb *P* elements are not shown to scale. (B) Expression of Ttk69 in stage 10b egg chambers. Ovaries were stained with an antiserum specific to Ttk69. Ttk69 is expressed in the follicle cells and reduced in *twk* egg chambers. (C) Western analysis showing the expression of Ttk69 in whole ovary extracts from wild type, two *ttk* allele combinations that display the twin peaks phenotype, and ttk^{l}, which complements ttk^{twk}. Ttk69 is reduced in ttk^{twk}/ttk^{twk} and ttk^{twk}/ttk^{1e11} ovaries but is normal in ttk^{l}/ttk^{l} ovaries. Immunoreactivity provided by antibodies against actin serves as a loading control.

morphological flaws. Normally, wild-type eggs are elongated, with distinct dorsal and ventral features. The ventral side of the egg is longer and more curved, while the dorsal side is marked by the presence of two long respiratory appendages (Fig. 1A). In contrast, eggs laid by *twk* females are small and round (Fig. 1B). An overall thin chorion results in fragile eggs that rupture easily. Finally, the dorsal respiratory appendages are reduced to rudimentary nubs that resemble the bases of wild-type dorsal appendages (Fig. 1B).

Closer examination of the dorsal appendage phenotype revealed a defect in dorsal follicle cell migration. During the first half of dorsal appendage morphogenesis, two groups of dorsal–anterior follicle cells move outward from the follicu-

TABLE 2
twin peaks/tramtrack Complementation Analyses

Genotype	Appendage phenotype			
	Wildtype	nubs	Other[a]	*N*
Canton S	453 (98%)	0	7 (1.6%)	460
twk/twk	0	201 (100%)	0	201
twk/Df(3R)awd-KRB	0	219 (100%)	0	219
twk/ttk^{1e11}	0	178 (100%)	0	178
twk/ttk^{1}	427 (88%)	0	55 (12%)	482
ttk^{1}/ttk^{1}	535 (90%)	0	59 (10%)	594
ttk^{1}/ttk^{1e11}	39 (40%)	3 (3.1%)	55 (57%)	97

[a] Other, short, thin, or fused dorsal appendages, or appendages of differing lengths.

lar epithelium (Dorman *et al.*, unpublished observations). This movement culminates in the production of a short tube visible as a wedge of follicle cells overlying the junction between the oocyte and the nurse cells at stage 12 (Fig. 1C). Next, this tube extends anteriorly to form a longer tube into which the follicle cells secrete the chorion proteins that make up the mature dorsal appendage (Fig. 1E). We examined each step of this process in *twk* mutant egg chambers to determine exactly when the defect occurs.

We employed differential interference contrast optics to examine ovaries from 2-day-old wild-type and *twk* females. Normally, the extent of dorsal appendage morphogenesis as well as the size of the oocyte contribute to egg-chamber staging criteria. Since eggs produced by *twk* females are small and have short dorsal appendages, these factors provided poor standards for judging *twk* mutants. We therefore staged egg chambers according to the following criteria: stage 10 egg chambers were those in which the oocyte occupied half the volume of the egg chamber; stage 10b egg chambers exhibited thickening of the dorsal anterior follicle cells relative to their neighbors. In stage 11 egg chambers, nurse-cell cytoplasmic transfer had begun as demonstrated by the clearing of yolk granules from the anterior of the oocyte. We defined subsequent stages by the extent of nurse cell apoptosis. At stage 12, at least 10 nurse cell nuclei remained in the egg chamber. By stage 13, only 2 to 3 nurse-cell nuclei remained, and by stage 14, no or 1 nurse cell nucleus could be observed (Spradling, 1993). This method of staging facilitated accurate comparison between wild-type and mutant egg chambers.

Analysis of dissected egg chambers revealed that the early stages of morphogenesis were normal in *twk* egg chambers: two groups of cells formed tubes visible as wedges (Fig. 1D). Tube extension began normally but stopped prematurely, frequently at the border between the oocyte and nurse cells. Note that wild-type follicle cells moved approximately twice as far as *twk* follicle cells (compare Fig. 1E with Fig. 1F). The *twk* follicle cells did not move farther than shown in Fig. 1D. Thus, the defect in *twk* mutant egg chambers was not in the initial phases of dorsal follicle cell migration,

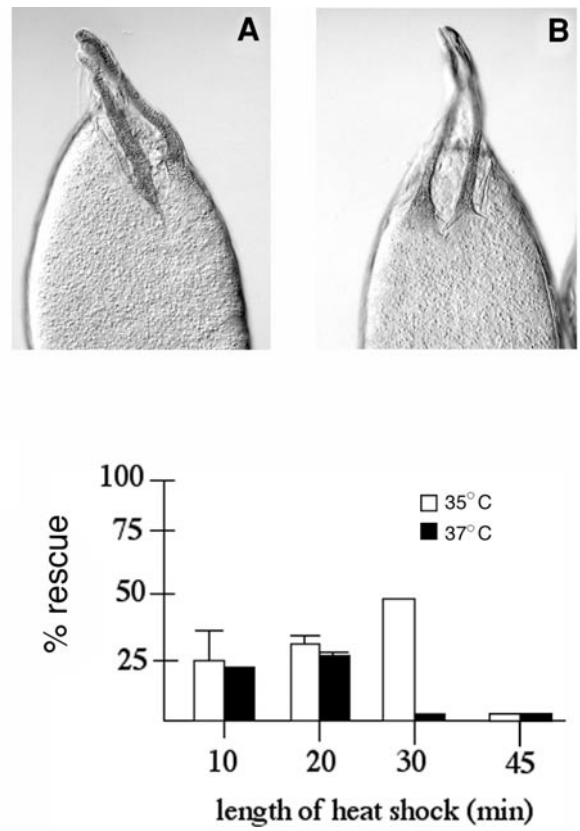

FIG. 5. Expression of Ttk69 in follicle cells rescues the twk morphogenetic defect. (A, B) Stage 14 egg chambers produced by females of genotype *w; hs-GAL4* ttk^{twk}*/UAS-ttk69* ttk^{lell}. The females were heat shocked for 30 min and allowed to recover for 20 h before ovaries were dissected. The egg chamber in (A) is partially rescued, while (B) demonstrates complete rescue (compare with Figs. 1A and 1B). (C) Quantitative data describing a variety of heat-shock rescue conditions. Percent rescue includes partially and completely rescued stage 13 and 14 eggs. Data points without error bars indicate an experiment was performed only once. The total number of eggs examined per experiment varied from 26 to 158 (mean = 80).

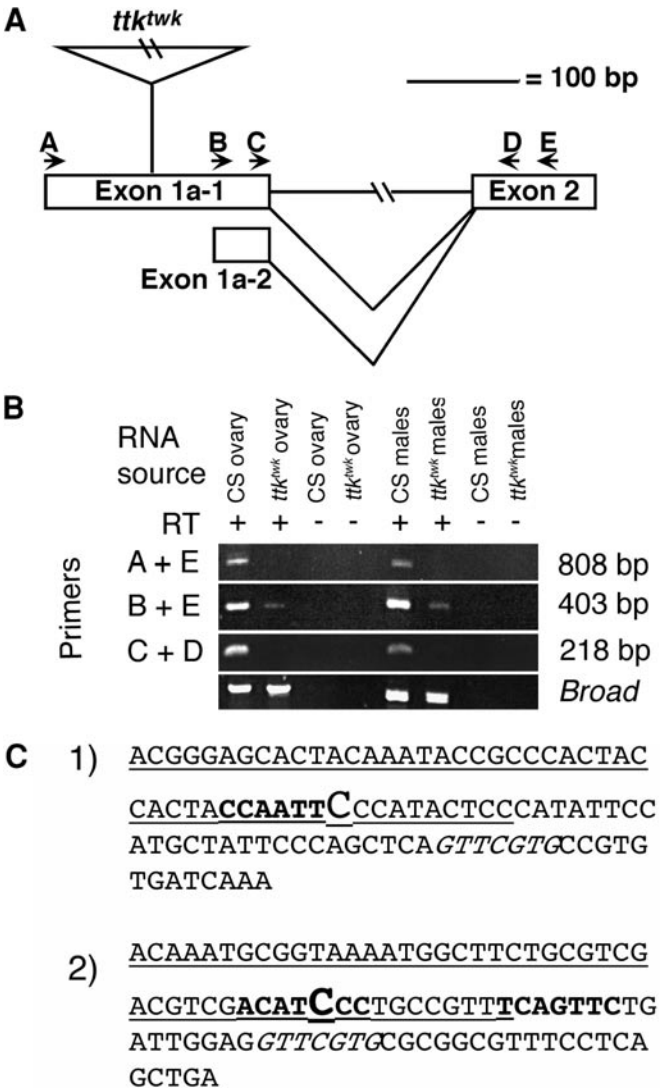

FIG. 6. Expression and promoter analyses of the ttk^{twk} region. (A) The locations of primers used in RT-PCR experiments relative to the *ttk* locus. Exon 1a-1 and exon 1a-2 indicate two potential transcriptional start sites. (B) RT-PCR analysis. All reverse transcriptase reactions were performed using primer E, except the positive control using primers specific for *Broad Z2*. RNA samples from wild-type (CS) and *twk* ovaries and adult males were compared by using primers located both 5′ and 3′ of the *P* element insertion. Each experiment was performed both with (+) and without (−) reverse transcriptase (RT). We detected transcripts specific to exon 1a-1 only in wild-type ovaries. Products containing either exon 1a-1 or exon 1a-2 (see the B + E reaction) were present in wild-type ovaries, *twk* ovaries and in males. In *twk* flies, it is unlikely that this band results from transcriptional read-through of the *P* element; thus, we hypothesize that transcription can initiate both upstream and downstream of the *P* insertion. Unexpectedly, this product was not detected using primers C + D in *twk* ovaries and wild-type males; variation in the ideal PCR conditions for these primers may have prevented detection of these rare transcripts. (C) Predicted *tramtrack* promoters. The BDGP promoter-prediction program identified the underlined sequences as possible promoters (Reese, 1998). Sequences in bold type represent potential initiator elements (InRs). Italicized sequences represent potential downstream promoter elements (DPEs). The predicted start sites for transcription are shown in larger type. 1, Potential-promoter 1 sequence begins 192 bases 5′ of the *twk* insertion site. 2, Potential-promoter 2 sequence begins 11 bases 3′ of the insertion site.

but occurred later, during tube extension. Table 1 shows quantitative data demonstrating the abrupt shift at stage 12 from wild-type to mutant phenotype in the *twk* egg chambers. We observed no defects in other follicle-cell migration processes, such as germ cell–cyst encapsulation, border cell migration, or centripetal migration.

twk Disrupts the Expression but Not the Amplification of Chorion Genes

In addition to the dorsal appendage defect, eggs laid by *twk* females were extremely fragile due to a thin chorion. Standard methods for dechorionating embryos, when applied to *twk* eggs, produced gelatinous blobs; these eggs exhibited no evidence of fertilization or subsequent embryonic development and lacked the vitelline envelope that normally helps retain shape (data not shown). Previous analyses by John Tower (personal communication) indicated that chorion-gene amplification was normal, but RNA levels from a subset of the chorion genes were reduced. We confirmed these results by analyzing the amplification and transcription of genes in both chorion clusters (Fig. 2).

The chorion is composed of 6 major structural proteins and approximately 15 minor proteins (Spradling, 1993; Waring and Mahowald, 1979). Because the eggshell is produced in a short (~5 h) period of time, the follicle cells must synthesize large amounts of the chorion proteins quite rapidly. In addition, the major chorion proteins are produced in a characteristic sequence: each gene is expressed for only a subset of the total time. Hence, chorion synthesis requires both rapid production of large amounts of protein as well as fine control over the timing of gene expression. These requirements are met in two ways. First, amplification of the two chorion gene clusters, beginning at stage 9, results in a 16- to 20-fold increase in X-cluster DNA content and a 60- to 80-fold amplification of the 3rd-chromosome cluster DNA. Second, precise transcriptional control of the individual chorion genes facilitates secretion of the chorion proteins in a temporal program that defines the various layers of the eggshell (reviewed by Orr-Weaver, 1991).

To assess chorion gene amplification, we extracted genomic DNA from adult flies and performed Southern analysis (Fig. 2A). In our previous studies on appendage morphogenesis, we established that ovaries from 1- and 2-day-old mutant females contained the same distribution of egg-chamber stages as wild type (data not shown). We

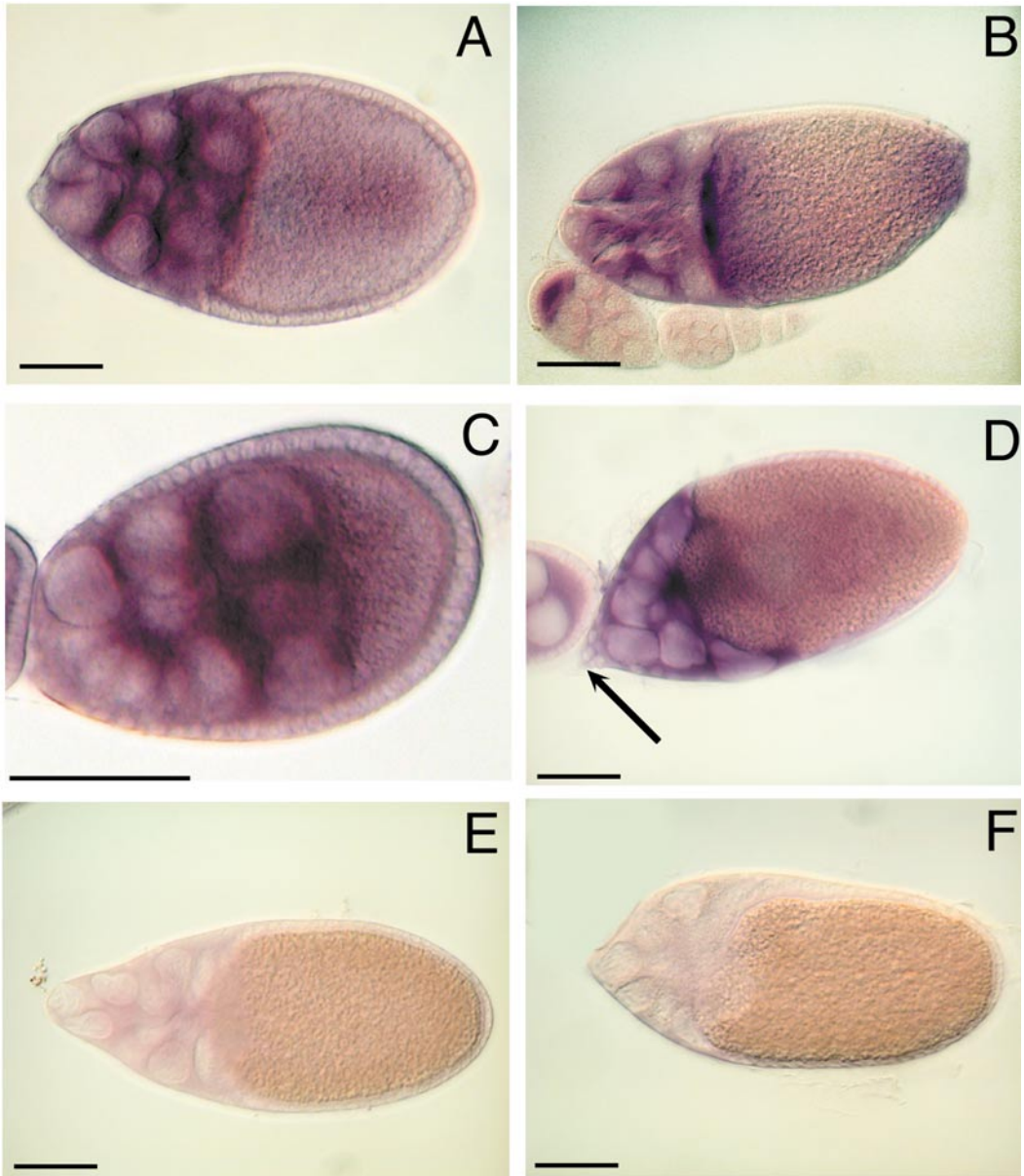

FIG. 7. *In situ* hybridization of alternative *ttk* first exons shows that only Exon 1a is expressed in the columnar follicle cells after stage 10 of oogenesis. (A) A stage 11 egg chamber hybridized with an antisense probe specific to exon 1a reveals that this exon is expressed in both germline and follicle cells. (B) A stage 11 egg chamber hybridized with a probe for *bicoid* serves as a negative control for follicle cell expression. (C, D) Stage 9 and late stage 11 egg chambers hybridized with an antisense probe for exon 1b show stable expression of this exon in the germline but dynamic expression in the follicle cells. In early stages (C), this exon is expressed in all follicle cells. Later (D), expression is absent in the columnar follicle cells but remains high in the stretch follicle cells (arrow). (E) An antisense probe for exon 1c shows no expression of this exon in oogenesis (compare with F). (F) No-probe control performed side-by-side with the exon 1c hybridization produces very faint stain.

probed genomic DNA from Canton S and *twk* females with a 4.4-kb genomic *Eco*RI fragment containing the X-chromosome gene *Cp36* (Spradling, 1981) or a 3.8-kb genomic *Sal*I fragment containing the third-chromosome genes *Cp15* and *Cp18* (Fig. 2A) (Lu and Tower, 1997). As expected, the third-chromosome fragment displayed a greater degree of amplification than did the X-chromosome fragment. Importantly, amplification of the X-chromosome fragment in *twk* females was 96% of that seen in wild type, and amplification of the third-chromosome fragment was

98% of wild-type levels. Thus, chorion gene amplification was not significantly affected by the *twk* mutation. Since chorion gene amplification in *twk* females was normal, we hypothesized that the chorion defect resulted from reduced mRNA levels for some or all of the chorion genes. To test this hypothesis, we conducted Northern analysis, using the same probes described above (Fig. 2B). Ovarian RNA extracted from *twk* females contained reduced amounts of at least two chorion gene transcripts. *Cp36*-transcript levels were reduced to approximately 47% of wild type, and the combined signal from the *Cp15* and *Cp18* transcripts was reduced to 28% of wild type. From these results, we conclude that the *twk* mutation results in decreased mRNA levels for at least two of the six major chorion protein genes. We hypothesize that the reduction in these transcripts in *twk* females results in the defective chorion phenotype.

Finally, to address the possibility that the reduction in chorion gene expression resulted from a general reduction in follicle cell transcription, we examined β-galactosidase expression in three enhancer-trap lines that are expressed in follicle cells (see Materials and Methods). The expression patterns of all three lines were normal in *twk* egg chambers (data not shown).

Several mechanisms could account for the dorsal appendage defects displayed by *twk* egg chambers: (1) *twk* mutants incorrectly specify the fate of dorsal–anterior follicle cells, (2) lack of chorion proteins disrupts tube elongation, or (3) *twk* mutants misregulate other factors that coordinate dorsal follicle movements.

We employed two approaches to distinguish among these possibilities. First, we analyzed two cell-fate markers to determine whether dorsal follicle cells establish and maintain a proper differentiation program. This analysis also revealed defects in cell movements. Second, we compared eggs produced by *twk* females with those produced by mutants known to directly alter chorion synthesis; this analysis tested whether normal chorion protein production is required for dorsal follicle cell morphogenesis.

The twk Defect Is Not Due to Improper Cell Fate Determination

Dorsal anterior follicle-cell fate is determined by the interaction of two signals, Egfr and Dpp (Dequier *et al.,* 2001; Peri and Roth, 2000). We tested whether the follicle cells had been correctly fated in *twk* egg chambers by examining the protein expression profile of Broad (Br), a zinc-finger transcription factor that responds to both Egfr and Dpp signaling during oogenesis. The *Br-Complex* is expressed in dorsal anterior follicle cells prior to and throughout their migration. These facts make Br an excellent molecule to test whether dorsal–anterior cell fates are established correctly in *twk* egg chambers.

Figure 3 shows the results of labeling wild-type and *twk* egg chambers with anti-Br antibodies. Early in oogenesis, Br is expressed in all columnar follicle cells (Deng and Bownes, 1997, and data not shown). In stages 10b and 11, the Br expression pattern refines in response to Egfr and Dpp signaling: levels of expression are higher in two patches of dorsal anterior follicle cells and much reduced on the dorsal midline and in several anterior rows of cells (Fig. 3A); high levels of expression persist in the dorsal–anterior follicle cells during tube formation and migration (Fig. 3B). Finally, near the end of oogenesis, expression remains high in the dorsal anterior cells and declines in all other follicle cells (Fig. 3C). We found that wild-type and *twk* staining patterns were indistinguishable at early stages of oogenesis (compare Fig. 3A with Fig 3E and Fig. 3B with Fig. 3F). After stage 12, we began to see differences in the arrangement of Br-expressing cells in *twk* egg chambers. The overall number of cells and the level of expression were similar, but these cells failed to move anteriorly (Fig. 3G). This phenotype was more consistent with a morphogenesis defect than a cell-fate defect. To quantify this result, we followed a method developed by Ellen Ward (E.J.W. and C.A.B., unpublished observations) that facilitates orienting, flattening, and imaging egg chambers. We counted the number of strongly Br-positive cells in each cluster of appendage-forming cells from stages 11 through 14, to test whether the correct number of cells adopted and maintained dorsal–anterior follicle cell fate in *twk* egg chambers. In wild-type egg chambers, we found 52.4 ± 6.2 strongly Br-positive cells per appendage at every stage of dorsal-appendage morphogenesis ($n = 23$, and E.J.W., unpublished observations); thus, the number of cells is consistent from stages 11 to 14. In addition, we counted 54.0 ± 6.8 strongly Br-positive cells per appendage at every stage in *twk* egg chambers ($n = 23$). These results suggest that improper cell fate decisions do not cause the *twk* dorsal appendage defect.

We next examined expression of Jun, the *Drosophila* homologue of the AP-1 transcription factor JUN (Perkins *et al.,* 1988). Jun is expressed in a subset of dorsal-appendage-forming cells and is required for several cell migration processes throughout development (Dobens *et al.,* 2001; Suzanne *et al.,* 2001; reviewed by Noselli, 1998). At stage 9 in oogenesis, Jun is expressed in all follicle cells (Dobens *et al.,* 2001, and data not shown). By stage 12, expression is elevated in the follicle cells over the nurse cells and in two groups of dorsal anterior follicle cells (Fig 3D). This pattern continues throughout dorsal follicle-cell migration (Dobens *et al.,* 2001, and data not shown). We found that expression of Jun in *twk* follicle cells resembled wild type at all stages of oogenesis (Fig 3H, and data not shown). These results lend support to our conclusion from the studies of Broad expression, that improper cell fate decisions do not cause the *twk* dorsal appendage defect.

The Migration and Chorion Production Defects Are Independent

Egg chambers produced by *twk* females exhibit defects in both cell migration and chorion synthesis. Importantly, some mutations that disrupt chorion gene amplification or expression result in thin eggshells with spindly or nub-like

dorsal appendages (Snyder *et al.*, 1986). These similarities led us to hypothesize that some aspect of chorion synthesis might be required for dorsal follicle cell migration. For example, chorion proteins might act as an extracellular matrix substrate for cell movement, or perhaps as the vehicle for some other extracellular signal. To test whether chorion production is required for dorsal follicle-cell migration, we examined cell movements in egg chambers from flies bearing mutations that disrupt eggshell synthesis.

The X-chromosomal gene *dec2* encodes the major chorion protein Cp36 (Mohler and Carroll, 1984), the first of the major chorion proteins to be expressed (Waring and Mahowald, 1979). Proper chorion assembly depends on the synthesis and secretion of Cp36 (Pascucci *et al.*, 1996). Thus, mutations in *dec2* result in severely defective eggshells. The defects displayed by *dec2* eggs are similar to those seen in *twk* eggs: the eggs are shorter and rounder than wild type, with a fragile chorion that is stripped away when the egg is laid (Fig. 3I, and data not shown). We reasoned that *dec2* mutants would be good candidates to test whether the structural integrity of the eggshell is a prerequisite for the anterior movement of dorsal follicle cells. To determine the extent of dorsal follicle cell morphogenesis in *dec2* egg chambers, we stained ovaries dissected from *dec2* females with anti-Br to identify the migratory cells. We found that *dec2* mutant follicle cells move outward over the nurse cells to generate tubes of approximately wild-type length (Figs. 3J and 3K). Although the organization of the Br-positive cells was less uniform (Fig. 3K), the distance traveled in all stage-14 egg chambers ($n > 50$) was 82 to 95% that of wild-type dorsal appendages. Finally, the chorion that was produced in *dec2* appendages appeared disorganized, and the dorsal appendages collapsed when the eggs were laid; thus, the mature dorsal appendages were smaller than predicted based on the length of the follicle cell tubes (Figs. 3I and 3J). These results suggest that chorion structural integrity is not required for dorsal follicle cell movement, at least at the level of resolution of this analysis. Therefore, it is unlikely that the *twk* dorsal appendage phenotype is due solely to reduced overall chorion production. It remains a formal possibility that a minor chorion protein not disrupted in the *dec2* mutant is responsible for the twk migration phenotype.

twin peaks Is a Unique Allele of tramtrack

The *twk* mutation is associated with a *P{lacZ; ry*$^{+}$*}* element, *07223*, inserted at 100D1-2 on the polytene chromosomes (data not shown). We employed plasmid rescue to recover a 23-kb fragment of genomic DNA flanking the insertion site. Sequence analysis of subclones from this 23-kb fragment revealed that *07223* was inserted into a region upstream of the published sequence of the gene *tramtrack* (*ttk*) (BDGP) (Fig. 4A). Further analysis demonstrated that the sequence immediately flanking the insertion site was contained within ESTs sequenced by the Berkeley Drosophila Genome Project, and that several of these ESTs were contiguous with known *ttk* cDNA sequences. The nearest gene proximal to this site mapped more than 28 kb 5′ to the *P* element (BDGP). We therefore hypothesized that the twk phenotypes were due to a disruption in *ttk*.

Through differential splicing, the *ttk* locus encodes two zinc finger proteins (Ttk88 and Ttk69) that function as transcriptional repressors (Brown *et al.*, 1991; Harrison and Travers, 1990; Read and Manley, 1992). The two proteins share a common BTB protein–protein interaction domain but have different sets of zinc fingers, which are encoded in the alternative final exons (see Fig. 4). As a result, they bind different DNA sequences and regulate different genes (Read and Manley, 1992).

The functions of both Ttk88 and Ttk69 in the developing nervous system have been well characterized, although somewhat more is known about the regulation of Ttk88. Both proteins are required in the embryonic peripheral nervous system (PNS) to repress neuronal cell fate (Guo *et al.*, 1995). In addition, Ttk88 is required to repress R7 cell fate in eye imaginal discs (Xiong and Montell, 1993), and much is known about its regulation in this system. In the future R1–R6 photoreceptor cells, *phyllopod* (*phyl*) transcription is induced in response to activation of the Ras/MAPK signal transduction pathway (Chang *et al.*, 1995; Dickson *et al.*, 1995). Phyl then forms a complex with Ttk88 and the ubiquitously expressed protein Sina, targeting Ttk88 for ubiquitin-mediated proteolysis (Li *et al.*, 1997; Tang *et al.*, 1997).

Unlike Ttk88, Ttk69 is required in the embryonic central nervous system (CNS), where it inhibits neuronal fate by repressing the transcription of the neuroblast-specific genes *asense* and *deadpan* (Badenhorst, 2001; Giesen *et al.*, 1997). Ttk69 also controls glial proliferation by inhibiting S-phase (Badenhorst, 2001). Additionally, in the eye imaginal disc, Ttk69 is thought to serve a dual role: it is first required to repress neuronal fate (Lai and Li, 1999), and subsequently required in the photoreceptor neurons themselves to maintain photoreceptor cell fate (Lai and Li, 1999; Xiong and Montell, 1993). Finally, in early embryogenesis, Ttk69 can repress transcription of several pair-rule genes, including *ftz*, *eve*, *hairy*, and *runt* (Brown *et al.*, 1991; Brown and Wu, 1993; Read *et al.*, 1992).

We employed several approaches to test whether the *twk* insertion disrupts *ttk* function. First, we generated excision alleles that reverted the twk phenotype. Second, we performed complementation analysis using deficiencies for the region and various *ttk* alleles. Finally, we rescued the mutant phenotypes using transgenic lines expressing Ttk69.

Excision Screen and Complementation Analysis

We generated 91 independent excision alleles of *twk* by mobilizing the *P* element with Δ2–3 transposase. Twenty-nine (31.9%) of these alleles produced completely wild-type eggs and displayed normal fertility. Thus, the *P{lacZ; ry*$^{+}$*}*

element was indeed responsible for the mutant phenotypes. Of the remaining excision lines, 27 (29.7%) were phenotypically indistinguishable from the original *twk* allele. Twenty-five lines (27.5%) displayed dorsal appendage and chorion defects, but these defects were significantly less severe than those of *twk* eggs. Finally, 10 lines (11%) laid no eggs; upon dissection, however, we found that the egg chambers produced by these females were similar to those produced by the original *twk* allele. We crossed all of these excision lines to a deficiency for *ttk*, and in all cases, the deletion failed to complement the oogenesis phenotype of the excision allele. Interestingly, although all previously known alleles of *ttk* reduce viability, we recovered no lethal alleles in our excision screen.

We analyzed DNA from 44 imprecise excision lines to determine the nature of the lesions. These lines included all 25 that partially reverted the twk phenotype as well as 19 lines that were phenotypically identical to *twk*. PCR analysis (using one primer that bound to the inverted repeats present at the ends of the *P* element, paired with another primer in the nearby genomic DNA) revealed that all 44 lines retained the 5′ end of the *P* element and the associated flanking DNA. This result may explain our failure to obtain lethal alleles, as the entire protein coding sequence of the *ttk* gene lies downstream of the 5′ end of the *P* element (Fig. 4). Six lines lacked the 3′ end of the *P* element. In these 6 lines, less than 200 bp of DNA had been deleted. We conclude from these analyses that the majority of our imprecise excision lines contained internal deletions of the *P* element.

Because our excision screen indicated that the *P* insertion into the *ttk* locus was responsible for the twk phenotype, we performed complementation tests using a variety of *ttk* alleles (Table 2). *Df(3R)awd-KRB* is a large deletion that removes the entire *ttk* locus as well as several neighboring genes downstream of *ttk*. The ttk^{1} mutation results from a *P*-element insertion into a region of overlap encoding an intron of a Ttk88-specific transcript and the 3′ UTR of a Ttk69-specific transcript (Fig. 4A). This allele predominantly affects production of Ttk88, probably by disrupting splicing of the *ttk88* mRNA, with no apparent effect on Ttk69 (Xiong and Montell, 1993). Consequently, this allele let us distinguish between a requirement for Ttk69 and Ttk88 in dorsal appendage morphogenesis. ttk^{lell} is an excision allele of ttk^{1} and deletes much of the last exon encoding Ttk69. This allele likely affects the production of both Ttk69 (by deleting the DNA binding domain) and Ttk88 (by disrupting splicing of the *ttk88* mRNA) (Fig. 4A).

Flies transheterozygous for *twk* and *Df(3R)awd-KRB* or any of the *ttk* alleles were fully viable relative to their heterozygous siblings. Analyses of eggshell defects, however, revealed a striking complementation pattern (Table 2). Egg chambers produced by ttk^{twk}/*Df(3R)awd-KRB* females were indistinguishable from those produced by females homozygous for *twk*. Both genotypes gave rise to short eggs with nub-like dorsal appendages and a severe chorion structure defect. Identical results were obtained with ttk^{lell}. In contrast, 88% of eggshells produced by females transheterozygous for ttk^{twk} and ttk^{1}, the allele that disrupts Ttk88, were wild type. The remainder of the eggs produced by ttk^{twk}/ttk^{1} females exhibited minor dorsal appendage defects that differed qualitatively from those produced by *twk* homozygotes. For example, some eggs had appendages of different lengths, and a small percentage had fused appendages. These data demonstrate that *twk* is indeed an allele of *ttk* and suggest that Ttk69 is the predominant protein required for chorion synthesis and dorsal appendage morphogenesis.

Ttk69 Is Required For Dorsal Appendage Morphogenesis

To test the hypothesis that loss of Ttk69 was responsible for the twk phenotypes, we employed antibodies raised against the zinc-finger domain of Ttk69 to examine expression in wild-type and mutant ovaries. Ttk69 is expressed in all follicle cells at all stages of oogenesis; this expression is diminished in *twk* ovaries (Figs. 4B and 4C), with the greatest effect on late stage egg chambers. We failed to detect Ttk88 in the ovary by immunofluorescence or Western analysis (data not shown); this result is consistent with previous studies (Read *et al.*, 1992). These data provide further evidence that Ttk69, and not Ttk88, is required for chorion synthesis and dorsal appendage morphogenesis.

We tested which of the Ttk protein isoforms could rescue the twk eggshell phenotype by individually expressing transgenic constructs using the GAL4/UAS system (Brand and Perrimon, 1993; Giesen *et al.*, 1997). Due to Ttk expression at earlier times in development, we failed to recover adult flies with most GAL4 lines tested (Ward *et al.*, 2002). We therefore generated females of genotype *y w UAS-ttk88/w; hs-GAL4 e* ttk^{twk}/ttk^{lell} or *hs-GAL4 e* ttk^{twk}/*UAS-ttk69* ttk^{lell} by raising the flies at 18°C. Our initial attempts at heat-shock-induced rescue using *hs-GAL4* and *UAS-ttk69* resulted in flies that were paralyzed and died within a few hours (data not shown). Consequently, we performed a series of heat shock experiments to identify appropriate conditions that allowed survival of the flies yet also rescued the twk eggshell phenotypes. Because *twk* dorsal appendages never extend beyond the nurse cells at the anterior end of the egg chamber, we defined partial rescue as stage 13 or 14 egg chambers with dorsal appendages that did extend past this point. We obtained a significant degree of partial rescue with short time periods and moderate temperatures (Fig. 5). Furthermore, these conditions resulted in partial rescue of the chorion defect: in dissected egg chambers, we observed chorion in the lumen of the developing dorsal appendages at stage 12. Chorion is never visible in the lumens of unrescued *twk* dorsal appendages (see Fig. 1D). Additionally, rare, laid eggs lacked the fragile-eggshell phenotype exhibited by *twk* eggs. Longer heat shocks resulted in a dramatic decline in rescue frequency (Fig. 5C) accompanied by an increase in defects early in oogenesis; these phenotypes likely resulted from

overexpression of Ttk69 (not shown). No rescue (or lethality) was observed in *y w UAS-ttk88/w; hs-GAL4 e* ttk^{twk}/ttk^{lell} under any set of heat shock conditions, nor did eggs laid by non-heat-shocked females display rescue (data not shown).

We dissected eggs from *hs-GAL4 e* ttk^{twk}/*UAS-ttk69* ttk^{lell} females subjected to a 30-min heat shock at 35°C (Figs. 5A and 5B). Rescued eggs were normal in shape and size, with dorsal appendages that were significantly longer than those observed in non-heat-shocked controls. At a low frequency, we observed dorsal appendages whose length and shape suggested complete rescue (Fig. 5B). These data, combined with the results of the complementation analyses and immunostaining, support the hypothesis that Ttk69 is required for dorsal-appendage morphogenesis and chorion synthesis, and that *twk* disrupts *ttk* function in the somatic follicle cells during oogenesis.

twk Does Not Mutate an Ovary-Specific Promoter

The *P* element responsible for *twk* is inserted into a previously unmapped *ttk* exon. This exon is found in cDNAs isolated from several libraries, including an adult head library (BDGP); thus, the transcript disrupted by the *twk* mutation must be expressed in tissues other than the ovary. Since *twk* does not affect viability, adult morphology, or behavior, we hypothesized that other *ttk* mRNAs can compensate for loss of the transcript disrupted by the *twk P* element. In addition, the ovary-specific phenotypes associated with *twk* could be due to mutation of a regulatory element that is specifically required for expression of *ttk* in oogenesis. We tested these hypotheses in three ways. First, we analyzed expression of the region surrounding the *twk* insertion by RT-PCR. Second, we carried out computational analysis of potential transcriptional start sites near the insertion point. Finally, we conducted *in situ* hybridization to assay for *ttk* RNA expression in ovarian tissue.

Primer extension analysis suggested that transcripts could initiate both 5′ and 3′ of the *P*-element insertion site in both ovaries and adult tissues (data not shown). Based on this result, we carried out RT-PCR analyses of the region surrounding the insertion site. In ovaries and adult males, transcripts were present that contained regions both upstream and downstream of the *P*-element insertion. These RNA species were reduced or abolished in *twk* mutant flies and ovaries (Fig. 6B). The presence of these transcripts in males confirms that *twk* does not disrupt an ovary-specific transcript; nevertheless, the phenotypes associated with *twk* are ovary-specific. We therefore hypothesize that the transcript(s) disrupted by the mutation, while not specific to oogenesis, is required specifically in the ovary. Loss-of-function in other tissues may be too subtle to detect with casual inspection, may require a specific behavioral or stress assay, or may be compensated by transcription from other *ttk* promoters.

Transcriptional Start Sites Exist Near the twk Insertion

To test whether the *twk* mutation disrupts a promoter required for expression of *ttk* in oogenesis, we used the promoter identification program provided at the Berkeley Drosophila Genome Project Web site to identify potential transcriptional start sites within the region neighboring the *twk* insertion. We identified two potential promoters within 900 bases of the *P*-element insertion site. The first of these potential promoters includes a start site for transcription approximately 150 bases 5′ of the insertion site, whereas the second transcriptional start site is predicted to be approximately 50 bases 3′ of the insertion site. The sequences surrounding both predicted start sites are consistent with TATA-less Drosophila promoters (Fig. 6C). Such promoters typically contain a downstream promoter element (DPE) with consensus sequence (A/G)G(A/T)CGTG at approximately +30 (Burke and Kadonaga, 1996). In addition, a second sequence, referred to as the initiator element (InR), with consensus sequence PyPyA+1NT, is often found near the transcriptional start site in both TATA-containing and TATA-less promoters (Javahery *et al.*, 1994). Sequences neighboring the *twk* insertion contained a good match to the DPE (GTTCGTG) 36–42 bases 3′ of the predicted upstream start site and 26–32 bases 3′ of the downstream site; sequences similar to the InR were present near both predicted start sites (Fig. 6C). In addition, 5 of 44 independent *ttk* 5′ EST sequences available from the BDGP contain sequence from Exon 1a; these sequences fall into two distinct categories. One set of ESTs begins approximately 150 bases upstream of the *twk* insertion, while the other begins approximately 50 bases downstream of the insertion site. These data support the hypothesis that two promoters reside within 200 bp of the *twk* insertion, and that one or both of these promoters is required for follicle-cell expression of *ttk*.

Exon 1a Is Required for Late Follicle Cell Expression of ttk

We analyzed 44 independent 5′ *ttk* cDNA sequences from the BDGP and discovered that each contained one of three, mutually exclusive, alternative-first exons. We hypothesized that these exons represented at least three alternative transcriptional start sites (Fig. 4A). The site of the *twk* insertion, combined with the follicle-cell specific phenotypes associated with the mutation, suggested to us that the first *ttk* exon was required for transcription of *ttk* in follicle cells, whereas the other start sites either were not used at all in oogenesis or specified a spatial or temporal expression pattern that did not provide the required Ttk69 function. This hypothesis, combined with our observation that the defects in *twk* egg chambers occurred after stage 11, led us to predict that of the three alternative first exons, only Exon 1a (see Fig. 4) would be expressed in the columnar follicle cells after stage 11 of oogenesis. To test this prediction, we

generated antisense RNA probes directed against the three alternative-first exons and performed *in situ* hybridization in ovaries (Fig. 7).

The results showed that *ttk* Exon 1a was expressed in all follicle cells as well as in the germline at all stages of oogenesis (Fig. 7A, and data not shown). For comparison, Fig. 7B shows the expression of *bicoid* mRNA, which is expressed in the germline but not in the follicle cells (Berleth *et al.,* 1988). A slightly different expression pattern was displayed by Exon 1b (Figs. 7C and 7D). Early in oogenesis, Exon 1b was expressed in the germline and at a low level in all follicle cells (Fig. 7C, and data not shown). By late stage 10, however, expression was no longer detectable in the columnar follicle cells covering the oocyte (Fig. 7D). Simultaneously, expression increased in the "stretch" follicle cells over the nurse cells (arrow in 7D). Finally, we observed no expression of Exon 1c above background levels (Figs. 7E and 7F), even after extensive incubation in detection reagent. We conclude that, during oogenesis, transcription beginning in exon 1c does not occur, or occurs at a level too low to be detected by *in situ* hybridization.

These results support the hypothesis that exon 1a provides essential *ttk* functions late in oogenesis and are consistent with the phenotypes observed in *twk* mutant egg chambers. Chorion gene expression and dorsal follicle-cell morphogenesis occur after stage 10; indeed, no defects are observed in *twk* egg chambers until stage 12. Furthermore, the third-chromosome chorion-gene cluster is expressed late in oogenesis (stages 13 and 14) (Griffin-Shea *et al.,* 1982), whereas the X-chromosome cluster is expressed somewhat earlier (beginning as early as stage 9 and accumulating until stage 12) (Parks *et al.,* 1986). This difference could explain the greater effect of the *twk* mutation on the transcription of genes from the third-chromosome cluster (Fig. 2). Finally, this expression profile is consistent with a requirement for Ttk69 in the columnar follicle cells. The columnar cells produce the eggshell, and a subset of these cells undergoes morphogenesis to form the dorsal appendages. Based on the promoter analysis, *in situ* hybridization data, and our observations of the twk phenotypes, we conclude that the *twk* mutation disrupts a transcript required in the columnar follicle cells after stage 10 of oogenesis.

DISCUSSION

We have identified a female-sterile allele of the gene *tramtrack, ttk*^{twk}, and shown that females homozygous for this allele produce egg chambers with several morphological defects. The most striking defect is a failure to complete dorsal anterior follicle-cell migration, leading to the production of short, nublike dorsal appendages. Female flies homozygous for *twk* also produce small, round eggs with a weak eggshell. This chorion defect causes the eggs to be fragile and easily ruptured, and is likely due to a severe reduction in transcript levels of at least one gene in the third-chromosome cluster of chorion genes, as well as a moderate reduction of transcription of the X-chromosome chorion-gene *Cp36*.

tramtrack and Chorion Gene Expression

While many known mutations affect chorion synthesis, nearly all reduce chorion gene amplification (Landis *et al.,* 1997; Schüpbach and Wieschaus, 1991; Snyder *et al.,* 1986). The proteins that regulate transcription of the chorion genes are not known. Two transcription factors that bind to the promoter of the chorion gene *Cp15* have been isolated (Shea *et al.,* 1990), but the functional significance of this binding has yet to be investigated. In *twk* egg chambers, chorion gene amplification is normal; nevertheless, these egg chambers produce extremely weak eggshells due to reduced mRNA from at least two of the major chorion genes. Ttk69 is consequently the only known protein required for the transcription of any of the major chorion genes.

The effect on chorion gene transcription could arise in two ways. Since Ttk69 is a transcription factor, it is tempting to speculate that the chorion genes are direct targets for transcriptional regulation by Ttk69. Alternatively, regulation of the chorion genes by Ttk69 may be indirect, a result of Ttk69 regulating factors upstream of chorion gene transcription. We favor this second hypothesis for two reasons. First, the hypothesis that Ttk69 directly regulates the chorion genes requires that Ttk69 function as a transcriptional activator. Currently, however, the only demonstrated function for Ttk69 is as a repressor of transcription (Badenhorst, 2001; Brown *et al.,* 1991; Brown and Wu, 1993; Read *et al.,* 1992, Xiong and Montell, 1993). Second, in our analyses of BDGP sequences, we found no matches to the Ttk69 consensus-binding sequence (Brown *et al.,* 1991) within 1 kb of any of the major chorion genes. Thus, it is more likely that Ttk69 regulates factors upstream of the chorion genes rather than the chorion genes themselves.

Intriguingly, the effect of the *twk* mutation on chorion gene expression is not uniform. While we see a reduction in the amount of at least two of the major chorion transcripts, the genes in the third-chromosome cluster are affected to a much greater degree than the X-chromosomal gene *Cp36*. This result is interesting in light of the exquisite temporal regulation of the individual chorion genes: although the genes are organized into clusters, each gene is expressed during a precise and unique window in time (Kalfayan *et al.,* 1985; Wakimoto *et al.,* 1986). Thus, further analysis of *twk* may shed light on the individual regulation of the chorion genes.

In addition to the defects in expression of the major chorion genes, we also observed an apparent vitelline envelope defect in *twk* eggs. It is therefore possible that Ttk69 is required for production of vitelline envelope proteins. Alternatively, this phenotype may reflect a requirement for one or more of the major chorion proteins in stabilizing the

vitelline envelope. Indirect evidence supports this latter hypothesis: until stage 12, Cp36, the earliest of the major chorion proteins, accumulates predominantly in the vitelline envelope (Pascucci *et al.*, 1996), and *dec2* mutant egg chambers lacking Cp36 display the same vitelline envelope defect exhibited by *twk* egg chambers (data not shown). Thus, interactions between chorion layers may be required for structural integrity of the eggshell (Pascucci *et al.*, 1996).

Ttk69 Is Required for Dorsal Appendage Morphogenesis

In wild-type ovaries, the dorsal anterior follicle cells undergo a characteristic series of cell shape changes and movements leading to the production of a tube of cells that secrete the chorionic appendages (Dorman *et al.*, unpublished observations). These events initiate normally in *twk* ovaries but arrest soon after the beginning of tube extension. Although Ttk69 is present throughout oogenesis, the loss-of-function phenotype is not evident until stage 12. This delay may be due to early transcription from exon 1b, which is not disrupted in *twk* ovaries (data not shown), combined with perdurance of the protein until stage 12. Alternatively, tube formation, which occurs normally in *twk* ovaries, and tube extension, which is defective, may be distinct processes under separate genetic regulation. Indeed, mutations in *kayak* (Fos), *hemipterous* (JNKK), *Jun related antigen (Jra,* Jun), and *puckered* (JNK phosphatase) all lead to the production of short dorsal appendages, in some instances very similar to *twk* (Dequier *et al.*, 2001; Dobens *et al.*, 2001; Suzanne *et al.*, 2001). These investigations demonstrate that proper regulation of the Jun kinase pathway is required for normal tube extension and support the hypothesis that the initiation of morphogenesis is a process distinct from tube extension. We considered the possibility that *ttk* might regulate the Jun kinase pathway in follicle cells, or vice versa. *twk* mutations do not alter the expression of Jun. Furthermore, strong alleles of *basket* (JNK) and *Jra (Drosophila* Jun) do not dominantly enhance the twk dorsal appendage phenotype (R.L.F. and C.A.B., unpublished results). These genes may act in separate pathways, both of which regulate tube extension. Alternatively, Ttk69 might act downstream of the JNK pathway and loss-of-function resulting from *twk* mutations is so severe that defects in other pathway members produce no discernable change in dorsal appendage morphology.

Ttk88 and Dorsal Appendage Patterning

Sequence analysis, complementation testing, immunohistochemistry, and transgenic rescue experiments demonstrated that *twk* is an allele of *ttk*. These studies strongly support the hypothesis that *twk* disrupts Ttk69 function. Furthermore, a mutation reported to specifically affect the expression of Ttk88 (ttk^{l}) does not cause twk-like dorsal appendage defects or chorion synthesis defects, either as a homozygote or in trans to *twk* (Table 2). Nevertheless, females homozygous for ttk^{1} do produce a small but significant number of egg chambers bearing aberrant appendages that are short, thin, or fused. A similar range of phenotypes is exhibited in egg chambers produced by females of the genotype ttk^{l}/ttk^{twk}. Furthermore, females of the genotype ttk^{l}/ttk^{lell} display a significant increase in these phenotypes. These results can be explained in several ways. First, it is possible that ttk^{l}, which is reported to affect only Ttk88 in other tissues, may actually exert a minor effect on the production of Ttk69 in the ovary. Alternatively, redundancy may exist between Ttk69 and Ttk88. Finally, Ttk88 may play a minor role in dorsal appendage patterning or morphogenesis. We favor the first alternative, since Ttk88 is undetectable in the ovary. It remains a formal possibility, however, that a low level of expression provides some function in patterning or morphogenesis of the dorsal anterior follicle cells.

Tramtrack Function in Oogenesis

The finding that *twk* is an allele of *ttk* is somewhat unexpected for several reasons. Of the more than 50 published alleles of *ttk*, nearly all are lethal; the rest display reduced viability. The lethality associated with mutations in *ttk* is attributed to several factors, including defects in the developing nervous system (Giesen *et al.*, 1997), failure of dorsal closure (Guo *et al.*, 1995), and other early embryonic defects (Xiong and Montell, 1993). We have isolated a completely viable but female sterile allele of *ttk*, ttk^{twk}, which reveals an unexplored function for Ttk69 during oogenesis.

The *P* element in *twk* is inserted into a 5′ exon that was previously uncharacterized. Our examination of EST data provided by the BDGP as well as computational analysis indicates that at least two promoters for the *ttk* gene may exist in this region, and that loss of transcription from one or both of these potential promoters leads to an oogenesis-specific defect. Our data indicate that neither of these potential promoters is specific to oogenesis; furthermore, *twk* disrupts transcription from this upstream region not only in oogenesis, but also in adult tissues. Nevertheless, transcription beginning in this exon is absolutely required for columnar follicle cell expression of *ttk* late in oogenesis. Presumably, alternative promoters can provide Ttk69 function in other tissues.

Ttk69 has been most extensively characterized in the developing embryonic nervous system, where it serves primarily as a bimodal cell fate switch, repressing transcription of genes required for determination of neuronal cell fate (Giesen *et al.*, 1997). In *twk* ovaries, however, dorsal anterior follicle cell fate is correctly determined. The expression patterns of *Broad*, which responds to both Grk and Dpp signaling (Deng and Bownes, 1997), and Jun, which is regulated by the JNK pathway (Dobens *et al.*, 2001), are completely normal in *twk* egg chambers. Thus, *ttk* must

function in dorsal anterior follicle cells after initial cell fate determination.

What, then, is the function of *ttk* in dorsal appendage morphogenesis? Assuming that Ttk69 functions as a transcriptional repressor in the ovary as it does in other tissues, we can speculate that a gene or genes must be repressed to allow tube extension to proceed. One simple hypothesis is that cell rearrangements or shape changes are necessary to allow part of the follicular epithelium to reorganize and extend forward over the nurse cells. Cell rearrangements might require the down-regulation of adhesion between cells, while shape changes might require altered levels of cytoskeletal proteins or apical/basal polarity determinants. Thus, it is tempting to speculate that one function of *ttk* in dorsal appendage formation is the repression of genes encoding regulators of cell morphology or adhesion.

With the isolation of *twk*, we have generated an additional tool with which to dissect the genetic regulation of dorsal appendage morphogenesis. We are examining the expression patterns of the known targets of Ttk69 to determine if any have a function in oogenesis. In addition, we are beginning genetic and molecular approaches to discover *twk* target genes in oogenesis. These studies will facilitate our efforts to understand the connection between patterning and morphogenesis in development.

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